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Smoky coal exposure, *NBS1* polymorphisms, p53 protein accumulation, and lung cancer risk in Xuan Wei, China

Qing Lan[a,*], Min Shen[a], Sonja I. Berndt[a], Matthew R. Bonner[a], Xingzhou He[b], Meredith Yeager[a,c], Robert Welch[a,c], Phouthone Keohavong[d], Mark Donahue[a], Pierre Hainaut[e], Stephen Chanock[a,c]

[a] *Occupational and Environmental Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, MSC 7240, 6120 Executive Blvd., EPS 8109, Bethesda, MD 20892-7240, USA*
[b] *Chinese Academy of Preventive Medicine, Beijing, China*
[c] *Core Genotyping Facility, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Bethesda, MD 20892, USA*
[d] *Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA 15260, USA*
[e] *International Agency for Research on Cancer, Lyon, France*

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Summary Lung cancer rates in Xuan Wei County are among the highest in China and have been associated with exposure to indoor smoky coal emissions that contain high levels of polycyclic aromatic hydrocarbons (PAHs). The *NBS1* gene product participates in DNA double-strand break repair and DNA damage-induced checkpoint activation, which are critical for maintaining genomic integrity. The *p53* tumor suppressor gene is known to play key roles both in the maintenance of genomic stability in mammalian cells and in DNA damage surveillance. We examined the association between two common *NBS1* polymorphisms (Leu34Leu, Gln185Glu) and lung cancer risk in a population-based case-control study in Xuan Wei, China. Individuals homozygous for the *NBS1* 34Leu or *NBS1* 185Glu variants were found to have an increased risk of lung cancer (odds ratio [OR] 2.15, 95% confidence interval [CI]: 0.91—5.10 and OR 2.53, 95% CI: 1.05—6.08, respectively). A haplotype containing the variant alleles from both *NBS1* SNPs was associated with increased risk of lung cancer compared with the most common haplotype. Further, the associations were particularly pronounced among cases with over expression of p53 protein. These results suggest that *NBS1* could be important in the pathogenesis of lung cancer in this population. However, additional studies in other populations with substantial environmental exposures to PAHs are needed to confirm our findings.

* Corresponding author. Tel.: +1 301 4354706; fax: +1 301 4021819.
E-mail address: qingl@mail.nih.gov (Q. Lan).

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1. Introduction

The lung cancer mortality rates in Xuan Wei County are among the highest in China for both males and females. Although almost all women do not smoke, mortality rates from lung cancer are similar to those for men, who have a high prevalence of smoking (27.7 and 25.3 per 100,000 for men and women in the county, respectively) [1]. Previous studies have shown that the etiology of lung cancer in Xuan Wei was primarily attributed to indoor exposure to smoky coal emissions, which have a high concentration of polycyclic aromatic hydrocarbons (PAHs) [1—3]. During the burning of smoky coal for home cooking, the indoor air concentration of benzo(a)pyrene (BaP), an indicator of PAHs, can become as high as 14.7 $\mu g/m^3$, comparable to exposure levels experienced by coke oven workers [1].

Studies have found that the activated form of BaP, benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), can bind to DNA at guanine positions of codons 153—158, 248, and 273 of the *p53* gene, form BPDE adduct, and cause DNA damage [4,5]. Stable covalent BaP DNA adducts can cause single-strand breaks which result in double-strand breaks (DSBs) during replication [6]. DNA DSBs are highly deleterious because they can be associated with chromosomal translocations and other aberrations [7]. The *NBS1* gene product participates in DNA double-strand break repair and DNA damage-induced checkpoint activation [8]. A cases only study [9] suggested an association between the *NBS1* Gln185Glu polymorphism and *p53* mutations, but the effect of common polymorphisms in the *NBS1* gene on lung cancer risk has not yet been examined in an epidemiologic case-control study.

Here, we evaluated the influence of two common *NBS1* polymorphisms on lung cancer risk, in a population-based, case-control study of residents in Xuan Wei County, China, in which exposure to PAH has been well documented [1,3]. Furthermore, we examined the joint association between the *NBS1* polymorphisms and p53 protein accumulation on lung cancer risk.

2. Materials and methods

This population-based case-control study has been described elsewhere [10,11]. Briefly, 122 lung cancer cases and 122 controls were enrolled between 1995 and 1996. The criteria for inclusion as a case for this study was based on histological or cytological evidence of lung cancer (105 cases) or death from lung cancer within a 1-year period (17 cases) [10,11]. Each control was matched to a case based on age (±2 years), sex, village and type of fuel currently used for cooking and heating at home. DNA was extracted from sputum samples and was successfully extracted from 119 case and 113 controls [10]. Genotyping was performed on an ABI 7900HT detection system using TaqMan endpoint reads as described on the website (http://snp500cancer.nci.nih.gov) [12]. We previously reported the association between p53 protein accumulation and smoky coal use [11]. Briefly, p53 protein accumulation was examined by immunocytochemical methods in exfoliated tumor cells isolated from sputum samples and has been described previously [11]. A series of five sputum samples were collected by spontaneous expectoration before surgery or any other type of treatment. Sputum was stored in 40 ml of Saccomanno's fluid [13]. The Saccomanno blending technique [13] was used to prepare the sputum samples prior to staining with Papanicolaou's reagent and p53 protein immunohistochemistry. Eight slides were made for each subject. The ABC ELITE mouse IgG detection kit (Vector Laboratories, Burlingame, CA) was used to detect p53 protein accumulation. The p53 immunostaining in sputum was evaluated by a scoring system for quantity and intensity [11]. Among subjects with the *NBS1* genotyping information, 93 had information on p53 protein accumulation.

The test for Hardy-Weinberg equilibrium among the controls was conducted using the observed genotype frequencies and a Pearson X^2 test with one degree of freedom. Logistic regression analysis was used to estimate the association between genotypes and lung cancer, adjusted for age, sex, and current fuel type. Further adjustment for smoky coal use and pack-years of smoking produced results that were very similar (data not shown). Therefore, we only presented the results that were adjusted for age, sex, and current fuel type. Tests for trends were conducted by including a single term for genotype status (0 for wild type homozygotes, 1 for heterozygotes, 2 for variant homozygotes) in a logistic regression model. The haplotype block structure was examined with HaploView (http://www.broad.mit.edu/personal/jcbarret/haploview/) using the four gamete rule [14] with a minimum frequency of 0.005 for the fourth gamete. Haplotypes were estimated using the Expectation-Maximization algorithm [15], and overall differences in haplotype frequencies between cases and controls were assessed using the omnibus test in SAS/Genetics (SAS Institute Inc., 2002). The association between each

Table 1 Demographic characteristics of lung cancer cases, by p53 protein accumulation status, and controls[a]

	Cases (n = 122)	Cases of p53 status (n = 97)		Controls (n = 122)	P-value[b]
	N (%)	Positive N (%)	Negative N (%)	N (%)	
Age					
<55	52 (43)	16 (36)	25 (47)	51 (42)	
≥55	70 (57)	28 (64)	28 (53)	71 (58)	0.90
Sex					
Male	79 (65)	33 (75)	28 (53)	79 (65)	
Female	43 (35)	11 (25)	25 (47)	43 (35)	1.00
Smoking[c]					
No	9 (11)	2 (3)	4(14)	10 (13)	
Yes	70 (89)	31 (97)	24(86)	69 (87)	0.81
Smoky coal use					
<130	51 (42)	20 (45)	23 (43)	72 (59)	
≥130	71 (58)	24 (55)	30 (57)	50 (41)	0.007

[a] The demographic characteristics of the study were previously reported [10,11]. Among the 122 cases and 122 controls, DNA was available for 119 cases and 113 controls. Among the 122 cases, 97 of them have information on p53 protein accumulation status.
[b] P based on chi-square test (all cases versus controls).
[c] Males only.

haplotype and lung cancer risk was estimated using the best haplotype pairs (i.e. the haplotype pair with the highest probability) for each person in a logistic regression model with the most common haplotype as the reference, adjusting for age, sex, and current fuel type.

The OR for smoky coal use was estimated as the average amount of lifetime cumulative tonnes of smoky coal. Smoky coal use was categorized into heavy and light based on the distribution in the controls. For subjects who used more than 130 tons of smoky coal in their life time defined as ''heavy'' smoky coal user and less than 130 tons as ''light'' smoky coal user. An ever-smoker was defined as a smoker of at least one cigarette per day for 6 months or longer. Gene environment interactions were examined and tested on a multiplicative scale by adding product terms into a logistic regression model. We stratified the association between *NBS1* and lung cancer by p53 protein accumulation status and calculated the adjusted odds ratios (ORs) and 95% confidence intervals (95% CI) in p53 positive and p53 negative lung cancer cases and controls. Polytomous logistic regression was used to compute ORs and 95% CIs for the p53 protein accumulation status analysis where the dependent variable had three levels: (1) p53 positive, (2) p53 negative, and (3) control [16]. Polytomous regression was analyzed by SPSS, version 12.0 (SPSS Inc., 2004). Data were analyzed with the Statistical Analysis Software, version 8.02 (SAS Institute Inc., 1996) if not otherwise specified.

3. Results

Table 1 shows demographic characteristics of lung cancer cases, by p53 status, and controls. Among cases and controls, type of fuel source, ethnicity, education level, household income and dwelling type were comparable (data not shown). Heavy smoking, namely, more than a 25 pack-year history was weakly associated with lung cancer risk (OR = 1.69; 95% CI: 0.82—3.49) in the men, a finding which is consistent with previous studies in Xuan Wei [1—3,10,17]. Compared to subjects who used less than 130 tons of smoky coal during their lifetime, heavy smoky coal users (those who used more than 130 tons) had a 2.27-fold (95% CI: 1.25—4.10) increased risk of lung cancer.

NBS1 genotype distributions were observed to be in Hardy-Weinberg equilibrium for the controls. The association of *NBS1* genotypes and haplotypes with lung cancer risk is presented in Table 2. Individuals homozygous for the 34Leu variant or 185Glu variant were found to have an increased risk of lung cancer. When stratified by smoky coal use or sex, the effect of the *NBS1* genotypes and the risk of lung cancer for both variants were not statistically different, but we had low power to detect such effects (data not shown). The two SNPs of the *NBS1* gene were in strong linkage disequilibrium ($D' = 1.0$) and nearly completely correlated ($r^2 = 0.99$), making it difficult to differentiate the effects of one SNP from the other on the risk of lung cancer. As expected, the haplotype containing the variant alleles from

Table 2 *NBS1*-Leu34Leu and -Gln185Glu polymorphisms and lung cancer risk

Genotypes and haplotypes	Case *N* (%)	Control *N* (%)	OR (95% CI)[a]	*P*-value
NBS1 Leu34Leu (Ex2 + 65G>A)				
rs1063045				
GG	37 (32.2)	46 (41.8)	Ref.	
GA	59 (51.3)	53 (43.6)	1.38 (0.78–2.44)	0.27
AA	19 (16.5)	11 (10.0)	2.15 (0.91–5.10)	0.08
GA + AA	78 (67.8)	64 (58.2)	1.51 (0.88–2.61)	0.14
Trend				0.07
NBS1 Gln185Glu (Ex5-32C>G)[b]				
rs1805794				
CC	37(31.4)	47 (42.3)	Ref.	
CG	61(51.7)	54 (48.6)	1.43 (0.81–2.52)	0.22
GG	20 (17.0)	10 (9.0)	2.53 (1.05–6.08)	0.04
CG + GG	81 (68.6)	64 (57.7)	1.60 (0.93–2.76)	0.09
Trend				0.03
NBS1 Haplotypes (Leu34Leu–Gln185Glu)				
Hap1 (G–C)	134 (56.8)	148 (66.1)	Ref.	
Hap2 (A–G)	101 (42.8)	75 (33.5)	1.48 (1.01–2.17)	0.04
Hap3 (A–C)	1 (0.4)	1 (0.4)	–	–
Omnibus test[c]				0.11

[a] ORs and 95% CIs obtained by logistic regression analysis adjusted for age, sex and current fuel type.
[b] CC genotype corresponds to the Gln/Gln genotype, CG to the Gln/Glu genotype, and *GG* to the Glu/Glu genotype.
[c] The *P*-value for the omnibus test based on permutations test.

both *NBS1* SNPs was associated with increased risk of lung cancer compared with the most common haplotype.

Table 3 shows the relationship between *NBS1* genotypes and haplotypes and lung cancer risk stratified by p53 protein accumulation status. The associations were particularly pronounced and significant only among cases that were p53 positive. However, the case–case comparison between p53+ and p53– cases was not statistically significant.

4. Discussion

We carried out a population-based case-control study of lung cancer in a uniquely exposed population in Xuan Wei County, China and observed an increased risk of lung cancer for individuals homozygous for both *NBS1* polymorphisms, 34Leu and 185Glu. Further, this association was particularly pronounced and statistically significant only among cases for whom p53 protein accumulation was detected in exfoliated tumor cells from sputum samples.

This study was conducted in a well-characterized population in which lung cancer has been associated with exposure to smoky coal emissions that contain high levels of BaP. BaP DNA adducts can cause single-strand breaks which may be converted to double-strand breaks upon DNA replication [6]. DSB can activate the checkpoint functions at the G1/S and the G2/M boundaries of the cell cycle. In response to double-strand breaks at the cellular level, NBS1, Mre11, and Rad50 form a complex at the site of DNA damage. The triple complex of NBS1, Mre11, Rad50 proteins displays enzymatic activities only when NBS1 is present. It has been shown that this complex is important for the regulation of genomic stability and could protect against malignant transformation [18]. Thus, the *NBS1* polymorphisms could affect the capacity of this complex to repair the damage caused by DNA adducts and increase the risk of cancer-causing mutations. We found that the *NBS1* (34A, 185Glu) variants were associated with an increased lung cancer risk, especially with p53 positive lung cancer. This suggests that NBS1 is important in preventing DNA mutations, such as *p53* mutations that disrupt the efficient repair of chromosomal abnormalities.

Mutations in the *p53* gene are critical events in the pathogenesis of cancer [19] and the most common genetic changes associated with human tumors, including lung cancer [20]. The *p53* tumor suppressor gene plays an essential role in maintaining genome integrity by influencing how the cell responds to DNA damage [21,22]. It can respond to DSBs by inducing cell cycle arrest, DNA repair,

Table 3 *NBS1*-Leu34Leu and Gln185Glu polymorphisms and lung cancer risk stratified by p53 protein accumulation status

Genotypes and haplotype	P53 status ($n = 93$)[a]		Control *N* (%)	P53 negative OR (95% CI)[b]	*P*-value	P53 positive OR (95% CI)[b]	*P*-value
	Negative cases *N* (%)	Positive cases *N* (%)					
NBS1 Leu34Leu							
GG	17 (35.4)	12 (28.6)	46 (41.8)	Ref.		Ref.	
GA	25 (52.1)	21 (50.0)	53 (48.2)	1.32 (0.63–2.76)	0.46	1.47 (0.65–3.34)	0.36
AA	6 (12.5)	9 (21.4)	11 (10.0)	1.37 (0.43–4.34)	0.59	3.38 (1.12–10.22)	0.03
GA + AA	31 (64.6)	30 (71.4)	64 (58.2)	1.32 (0.65–2.69)	0.44	1.78 (0.82–3.86)	0.15
Trend					0.48		0.04
NBS1 Gln185Glu[c]							
CC	17 (34.7)	12 (27.3)	47 (42.3)	Ref.		Ref.	
CG	25 (51.0)	22 (50.0)	54 (48.6)	1.30 (0.62–2.71)	0.49	1.57 (0.70–3.53)	0.28
GG	7 (14.3)	10 (22.7)	10 (9.0)	1.82 (0.59–5.59)	0.30	4.21 (1.40–12.66)	0.01
CG+GG	32 (65.3)	32 (72.7)	64 (57.7)	1.38 (0.68–2.79)	0.37	1.96 (0.91–4.22)	0.09
Trend					0.28		0.02
NBS1 haplotype analysis (Leu34Leu—Gln185Glu)							
Hap1 (G—C)	59 (60.2)	46 (52.3)	148 (66.4)	Ref.		Ref.	
Hap2 (A—G)	39 (39.8)	42 (47.7)	75 (33.6)	1.28 (0.78–2.09)	0.33	1.83 (1.10–3.03)	0.02
Hap3 (A—C)	0	0	1 (0.4)	—	—	—	—
Omnibus test[d]					0.50		0.02

[a] Ninety-three of the patients had information on p53 protein accumulation.
[b] ORs, 95% CIs, and *P*-values obtained by polytomous logistic regression analysis adjusted for age, sex, current fuel type.
[c] CC genotype corresponds to the Gln/Gln genotype, CG to the Gln/Glu genotype, and GG to the Glu/Glu genotype.
[d] The *P*-value for the omnibus test based on permutations test.

or apoptosis. Studies have shown that the specific mutation patterns of GC to TA transversion in *p53* gene are the most commonly observed mutations following experimental exposure to polycyclic aromatic hydrocarbons, such as B(a)P [23]. In a previous study, we found that *p53* was mutanted in 17/24 (71%) of nonsmoking female cases who used smoky coal in Xuan Wei. In these patients, a total of 21 mutations were identified (four patients having two distinct mutations), including 16 (76%) GC to TA transversions, the majority of them occurring at positions experimentally identified as common sites of adduct formation by metabolites of PAH [4]. Detection of p53 protein accumulation in cytology specimens from sputum is a good surrogate marker for the presence of a mutation. Indeed, in a pilot study on 15 of the 97 cases, we observed that *p53* and *K-ras* gene mutations, consisting predominantly of GC to TA transversions, were detected in tumor cells isolated from sputum of these patients [24]. However, in our study we used an immunohistochemical method to assess p53 protein expression rather than directly sequencing p53 mutations, as only sputum samples were available from the cases. As a consequence, the findings should be interpreted with caution as they may not necessarily apply to lung cancer cases categorized by *p53* mutation pattern.

We found that the *NBS1* (34A, 185Glu) variants were associated with an increased lung cancer risk, especially with p53 positive lung cancer. It is also possible that the *NBS1* (34A, 185Glu) variants may be more efficient than the wild-type *NBS1* alleles in activating wild-type *p53* after strand break DNA damage. The result of this activation would be to suppress the proliferation of cells with wild-type *p53* (either through growth arrest or apoptosis), whereas cells that have acquired a *p53* mutation would escape suppression and thus have a selective advantage for tumorigenesis. According to this hypothesis, PAH exposure would cooperate with *NBS1* (34A, 185Glu) variants in a mechanism of Darwinian selection of cancer cells, with PAH as the mutagen and *NBS1* (34A, 185Glu) variants as the target for selection pressure.

To our knowledge, no information is available regarding the effect of the *NBS1* Leu34Leu and Gln185Glu polymorphisms on DNA repair activity. The Gln185Glu polymorphism could have functional significance, because the amino acid substitution is a non-conservative change, but further studies are needed to characterize this. Although it is also possible that the synonymous SNP at 34Leu could alter stability of messenger RNA [24], it is more likely that the association that we observed between this allele and lung cancer is due to linkage disequilibrium with the 185Glu allele. Alternatively, the positive association we observed between these two polymorphisms and lung cancer could be due to an unobserved variant that is in linkage disequilibrium with both SNPs.

Medina et al [9]. reported that the *NBS1* 185Gln variant was associated with a significantly higher number of *p53* mutations among lung cancer patients in a case-only study. In contrast, we found that the *NBS1* 185Glu variant was associated with p53 protein accumulation. This discrepancy between our study and the study by Medina et al. could be due to differences in ethnicities of the study populations as well as in differences in exposures. The study by Medina et al. included both blacks and whites, whereas our study consisted of only Chinese subjects. The frequency of the *NBS1* polymorphism in the control population from our study in China was substantially lower than that observed in Caucasian populations [9], consistent with data from the NCI SNP500 Cancer re-sequencing project (http://snp500cancer.nci.nih.gov) [14]. It is possible that if the observed association between the *NSB1* polymorphism and p53 status is due to linkage disequilibrium with an unknown variant, the linkage patterns and thus associations with risk may differ between ethnic populations.

A key strength of our study is that it used a population-based case-control design and had very high participation rates. Further, most lung cancer cases were caused by environmental exposure to smoky coal, which contains high levels of PAH, instead of tobacco smoke. However, our study has a small sample size and as a consequence low statistical power to detect effects, and an increased possibility of having false-positive findings [25]. As such, our findings, while intriguing, should be considered preliminary until they can be evaluated in a larger study. Also, a more extensive analysis of *NBS1* to comprehensively assess the contribution of genetic variation across the gene [26] to lung cancer risk is needed, as well as studies examining the impact of common genetic variation on in the *NBS1* gene on DNA repair activity. Further, a larger hospital-based case-control study of lung cancer in this relatively unique population will be started this year and will provide an opportunity to confirm these findings.

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